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Steroid hormones are involved in the development and growth of the breast cancer. Drugs which inhibit estrogen action are commonly used to inhibit breast cancer growth. Unfortunately, most advanced breast cancer become resistant to estrogen treatment. Recently, many steroid receptor coactivators have been discovered and found to potentiate the transcriptional activity of steroid receptors and enhance the expression of hormone response genes. SRC-1 family of coactivator, AIB1 is found amplified and/or over-expressed in breast cancer specimens. To evaluate the potential roles of SRC-1 family of coactivators in mammary tumorigenesis *in vivo*, we proposed to generated transgenic mice over-expression of AIB1 (SRC-3) in mammary glands. To target the expression of AIB1 in mammary gland, we placed the AIB1 transgene under the control of the MMTV-LTR promoter. Two lines of transgenic mice expressing AIB1 have been generated. Studies on these transgenic mice will help understanding the development and progression of breast cancer and provide molecular basis for design novel strategies to curb and ultimately cure breast cancer.

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Stephen Y. Pai 4/28/2000

PI - Signature Date

Table of Contents

INTRODUCTION

Breast cancer is the most prevalent disease among women in the United States. It is well documented that estrogen stimulates mammary epithelial cell growth and increases the incidence of breast cancer. Hormone ablative therapy has been successfully used to inhibit estrogen receptor (ER) dependent growth of breast cancer. However, cell growth of most breast cancers subsequently becomes resistant to anti-estrogen treatment. The mechanism underlying the progression of breast cancer growth from hormone-dependent to hormone-independent state is largely undefined.

Recently, many steroid receptor coactivators have been discovered and found to stimulate the transcriptional activity of steroid receptors and enhance the expression of hormone response genes. Steroid receptor co-activator-1 (SRC-1) family of coactivators appeared to be closely involved in mammary gland development and tumorigenesis. The mammary ductal arborization and alveolar formation in response to estrogen and progesterone are severely compromised in the SRC-1 null mice. Another member of SRC-1 family, AIB1, is found to be amplified in 10% of breast cancer patients and it is over-expressed in 64% of breast cancer specimens. Given these compelling evidence and the fact that this family of coactivators is essential for the synthesis of hormone regulated genes, we hypothesize that deregulated expression of these factors may play an important role in the initiation and progression of mammary tumorigenesis. To test our hypothesis, we proposed to generate transgenic mice over-expression of AIB1 (SRC-3) in mammary glands, and examine whether unwarranted expression of these co-activators will result in mammary tumorigenesis.

BODY

Generation of MMTV-AIB1 transgenic construct

A BamHI-SalI fragment of the KCR vector containing part of exon II, intron II, exon III and the 3' untranslated region of the rabbit betaglobin gene, sequences spanning the SV40 polyA and parts of the pBR322 plasmid sequences were cloned into corresponding sites of the bluescript vector KSII from Stratagene. The use of the KCR fragment is to facilitate proper splicing and expression of the regulator transgene. A 2.3 Kb BamHI fragment of the MMTV-LTR was subsequently cloned into the BamHI site of the KCR to construct MMTV-KCR expression vector. To facilitate mammary gland-specific expression, AIB1 is put under the control of the MMTV promoter. The blunt-ended Acc65I-SalI 4.5 kb full-length Flag-tagged/HA-tagged AIB1 cDNA was subcloned into the EcoRI blunt-ended site of MMTV-KCR vector to obtain the MMTV-AIB1 intermediate vector. A 2.4 kb insulator fragment (HS4) was placed upstream to the resulting MMTV-AIB1 construct to obtain the HMAIB1 construct with the hope to reduce the position effect of integration sites on transgene expression. (Fig 1A).

Generation of MMTV-AIB1 transgenic mice

The linearized 11 kb NotI-Acc65I MMTV-AIB1 fragment was microinjected into the fertilized one-celled FVB embryos and the resulting viable embryos transferred into psuedopregnant FVB female recipients to allow the embryos to develop to term. The potential

founder pups born were allowed to develop to weaning age (~21 days) and were then genotype by Southern and PCR analyses.

34 pups were born and screened by first PCR amplification of transgene from purified tail DNA using forward primer 5'-ATTCCTCCTTGACCAACTCC-3' and reverse primer 5'-TCCTGCTTGACAACATTTCC-3'. Through the initial PCR screening, 9 founder lines were obtained. The transgenic founder were further confirmed by Southern analysis of BamHI and BglII digested DNA from the founder lines. A representative Southern analysis is depicted in Figures 2. A probe derived from part of the AIB1 cDNA, the Acc65I-ClaI portion, was used for hybridization . The expected bands of 5 kb were obtained in the Southern analysis.

Expression of AIB1 in mammary glands of transgenic mice

Each of the transgenic lines were analyzed for the expression of AIB1 transgene using RNase protection assay (RPA). To generate the antisense probe for RNase protection assay, an AIB1 cDNA fragment was amplified by PCR using following primers:
5'- CGGGATCCTCTCAACCCACTTCCTTC-3' (Forward)
5'-GCTCTAGACATACCTAGCTCCACTCATC-3' (Reverse)
The resultant PCR fragment was digested with BamHI and XbaI and subcloned into corresponding sites of pBS-KS plasmid (Strategene). ^{32}P labeled antisense probe was synthesized by in vitro transcription using T7 RNA polymerase. RNA was isolated from mammary gland biopsies of transgenic and non-transgenic littermates at 10-days lactation whereby the MMTV promoter has been shown to be highly active. RPA was performed by hybridizing AIB1 antisense probe with mammary gland RNA and yields a 210 bp protected fragment (see Figure 3). The antisense cyclophilin probe was included in each sample as a reference standard. As shown in the autoradiogram in Figure 3, the AIB1 antisense probe is specific for AIB1 transgene and do not cross-react with endogenous mouse mP/CIP gene (human AIB1 homologue). Transgenic lines 2649 and 9845 were found to express AIB1 transgene.

Tissue specific expression of AIB1 transgene in transgenic mice

To determine the tissue specificity of transgene expression, total RNA isolated from a variety of transgenic tissues of 9845 transgenic line were used for RPA analysis using AIB1 antisense probe. An cyclophilin riboprobe was used to quantitate loading. As expected, The transgene is abundantly expressed in mammary glands. Lower level of AIB1 expression is also detected in many other tissues such as salivary gland, spleen, lung and hypothalamus (Figure 4). These results show that the targeting of AIB1 transgene to the mammary gland is fairly specific and resembles the normal pattern of MMTV-directed expression. Analyses of the other transgenic line 2649 are underway.

Expression of endogenous mouse P/CIP in transgenic mice

To check whether AIB1 transgene is over-expressed in transgenic mammary gland we measured the expression of endogenous mouse P/CIP (human AIB1 homologue) in mammary

gland and others tissues by RPA analysis. To generate the antisense probe for RNase protection assay, an mP/CIP cDNA fragment was amplified by PCR using following primers:
5'- CGGGATCCGTTCTGACTTCTACAACAATCC-3' (Forward)
5'-GCTCTAGACGTAATTCTCCTGACTATCC-3' (Reverse)
The resultant PCR fragment was digested with BamHI and XbaI and subcloned into corresponding sites of pBS-KS plasmid (Strategene). ^{32}P labeled antisense probe was synthesized by in vitro transcription using T7 RNA polymerase. Total RNA isolated from a variety of transgenic tissues of 9845 transgenic line were used for RPA analysis using mP/CIP antisense probe. An cyclophilin riboprobe was used to quantitate loading. 260bp protected fragment represent the endogenous mP/CIP mRNA. As shown in Figure 5, mP/CIP antisense probe is very specific. No cross-reaction with human AIB1 was found in control RNA sample isolated from MCF-7 cells. Mouse mammary gland expressed low level of mP/CIP. mP/CIP is also expressed in many other tissues. mP/CIP expression is relatively high in heart, lung spleen and uterus. By comparison with their relative signal to cyclophilin loading control, expression level of AIB1 transgene is about 2 to 3 fold higher than that of endogenous mP/CIP expression in mammary glands.

Modification of MMTV-AIB1 expression vector

In the effort to achieve higher levels of expression of AIB1 transgene in mammary gland, we chose another MMTV-SV40 expression vector that is widely used for targeting the expression of transgenes in mammary glands. The blunt-ended Acc65I-SalI 4.5 kb full-length Flag-tagged/HA-tagged AIB1 cDNA was subcloned into EcoRI blunt-ended site of MMTV-SV40 expression vector Figure 1B. Similarly, the linearized 8.8 kb SalI-AseI MMTV-AIB1 fragment was microinjected into the fertilized one-celled FVB embryos and the resulting viable embryos transferred into psuedopregnant FVB female recipients to allow the embryos to develop to term. The potential founder pups born were allowed to develop to weaning age (~21 days) and were then genotyped by Southern and PCR analyses. Of 55 pups born, 7 pups were shown to carry AIB1 transgene by PCR analysis. However, none of these transgenic founders were shown to express AIB1 transgene in mammary gland as analyzed by RPA analysis. Table 1 shows our attempts to generate the AIB1 transgenic mice and the expression lines of AIB1 transgenic mice we obtained so far.

Ongoing Experiments

Analysis of mammary gland phenotypes of transgenic mice expressing AIB1

To analyze for physiological perturbations that could ascribed to the -expression of AIB1 in transgenic lines 9845 and 2649, we will perform whole-mount analysis and histological examinations on the transgenic mammary glands. Mammary glands at different stages of development, i.e. virgin, pregnancy, lactation and involution stages will be used for evaluation of ductal and alveolar morphogenesis. Since AIB1 acts as steroid receptor coactivator, the expression of steroid receptor responsive genes will also be analyzed through Northern blot or RPA analyses.

Generation of transgenic mouse lines over-expressing AIB1 to higher levels in mammary glands

It is imperative to generate transgenic mouse over-expressing AIB1 in mammary glands. To this aim, we will continue to microinject the transgenic construct and generate more lines of the MMTV-AIB1 transgenic mice.

Multiple factors might contribute to low expression of AIB1 transgene. It is possible that mammary epithelial cells might not tolerate high levels of AIB1 expression since AIB1 is a potent coactivators. To get around that problem, we try to use the regulable system that developed in our lab to turn-on the expression of AIB1 expression at specific time point. Figure 6 depicts this modified strategy to target the expression of AIB1 in mammary at high levels. AIB1 transgene will be placed under the control of four yeast transcription factor GAL4 binding sites (refereed as 17X4). Transgenic mice (also named target mice) carrying this transgene will be crossed with regulable mice generated previous in this lab. When these two transgenic lines are crossed to create the bitransgenic mice, the regulator can then bind to the 17 x 4 UAS recognition sequences upstream of the target oncogene and induce the expression of AIB1 transgene with the administration of RU486. The expression of AIB1 in the bitransgeneic mice can be turned-on by RU486 at a specific window during development and the effects of this coactivator on mammary gland oncogenesis can be monitored.

KEY RESEARCH ACCOMPLISHMENTS

1. Generated two MMTV-AIB1 transgenic constructs.
2. Constructed plasmids transcribing specific AIB1 and mP/CIP antisense probes.
3. Established two lines of transgenic mice expressing AIB1 transgene predominantly in mammary glands.

REPORTABLE OUTCOMES

1. Established two lines of transgenic mice expressing AIB1 transgene in mammary glands.

CONCLUSIONS

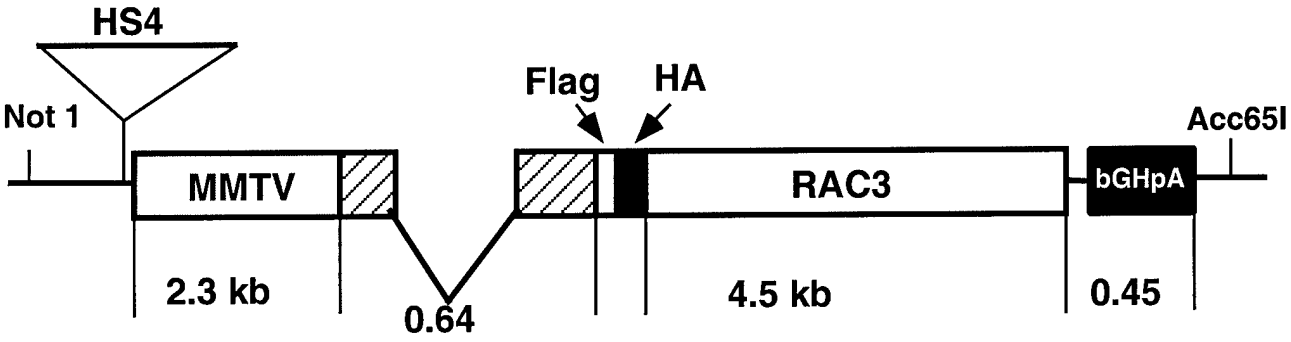
We have generated two transgenic lines expressing AIB1 transgene in mammary glands. Tissue specific expression analysis indicates that line 9845 expresses AIB1 transgene predominantly in mammary glands. The expression level of AIB1 transgene is about 2 to 3 fold higher than endogenous mouse map/CIP expression. We are currently characterizing the mammary gland phenotypes of these transgenic mice at different development stages. Studies on these transgenic mice will gain insight into the breast cancer formation and progression and provided molecular basis for design novel strategies to curb and ultimately cure breast cancer.

APPENDICES

Figures and Table

MMTV-AIB1 Transgene Constructs

A. HMAIB1 construct:



B. MSAIB1 construct:

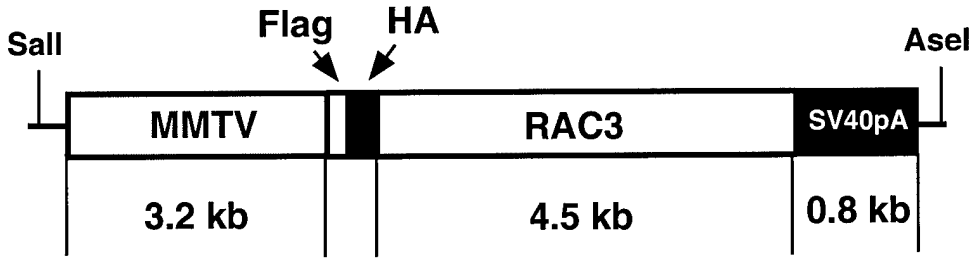
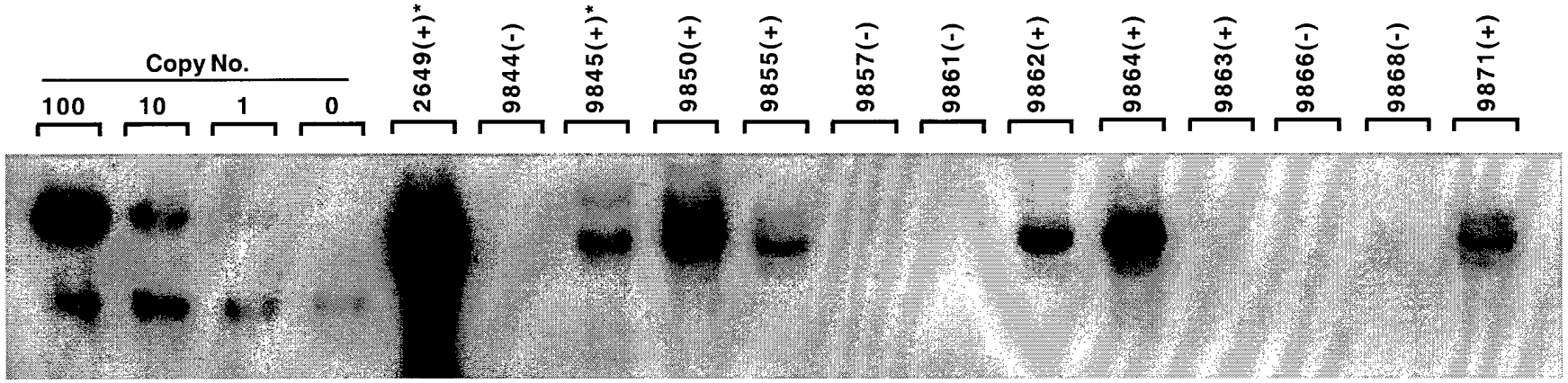


Figure 1

Southern Blot Analysis of MMTV-AIB1Transgenic Founder Mice



* : Lines expressing AIB1 in mammary glands

Figure 2

Expression of AIB1 Transgene in Mammary Glands of Transgenic Founder Mice

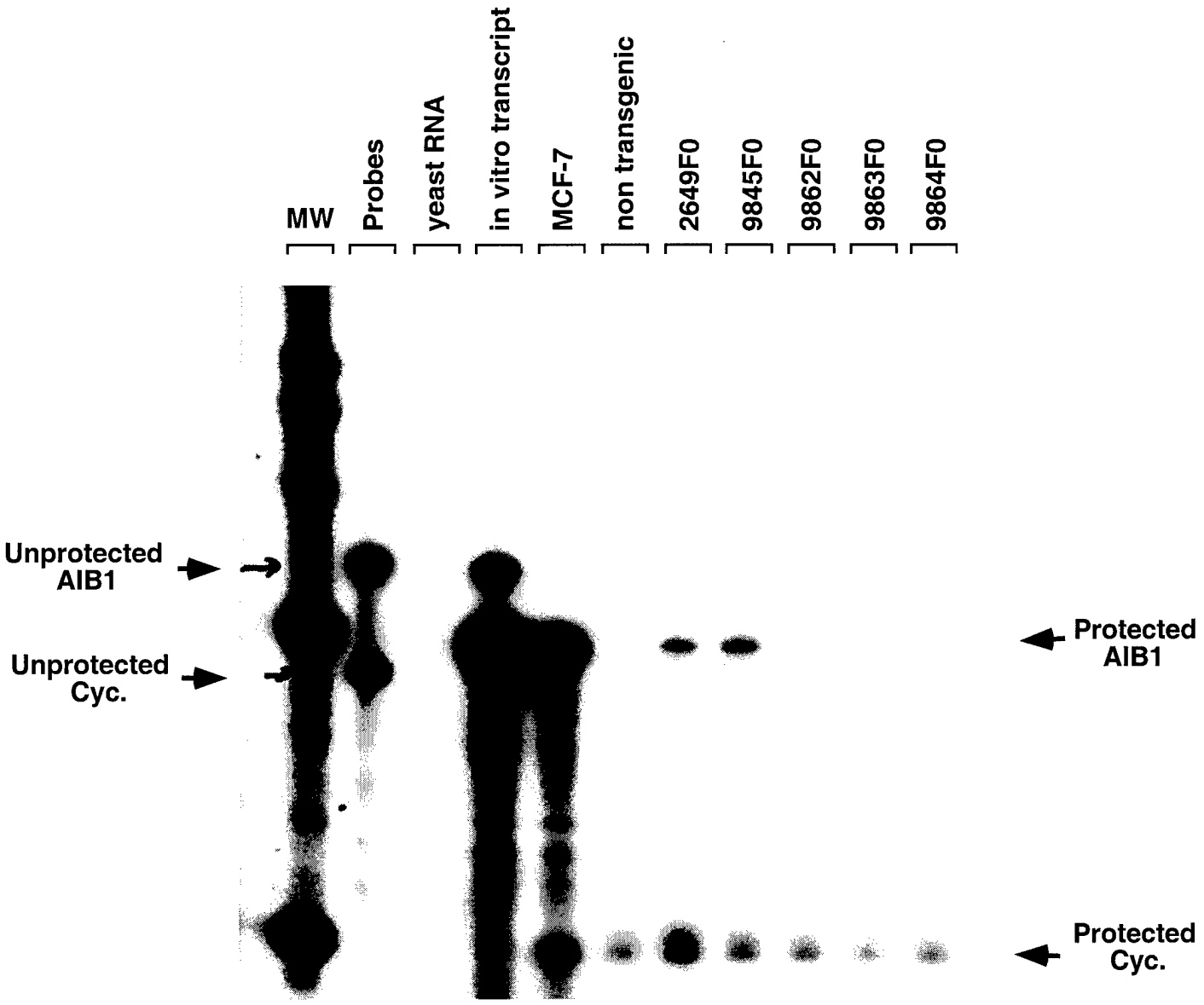


Figure 3

Tissue Specific Expression of AIB1 Transgene in Transgenic Mice

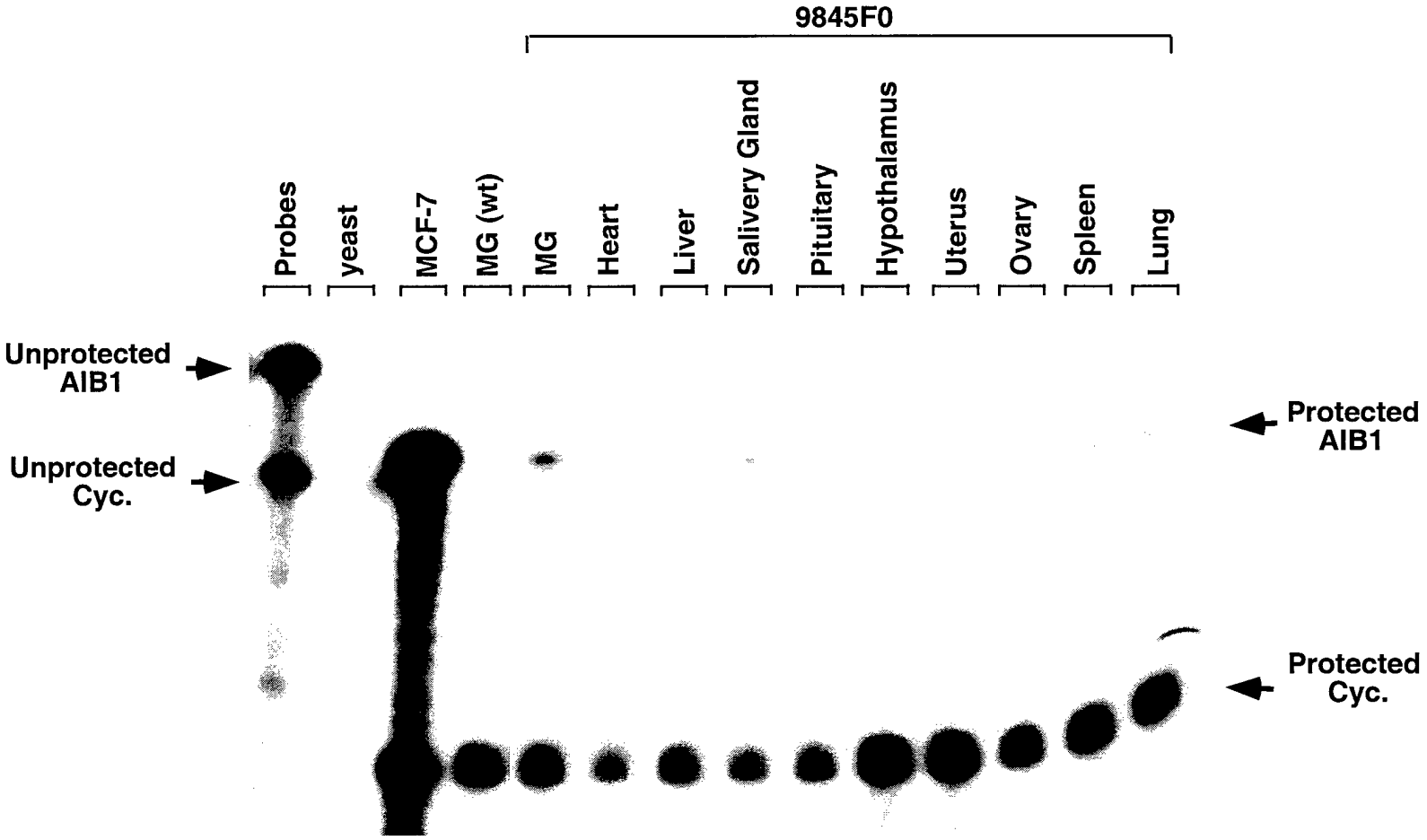


Figure 4

Expression of Endogenous Mouse P/CIP in Transgenic Mice

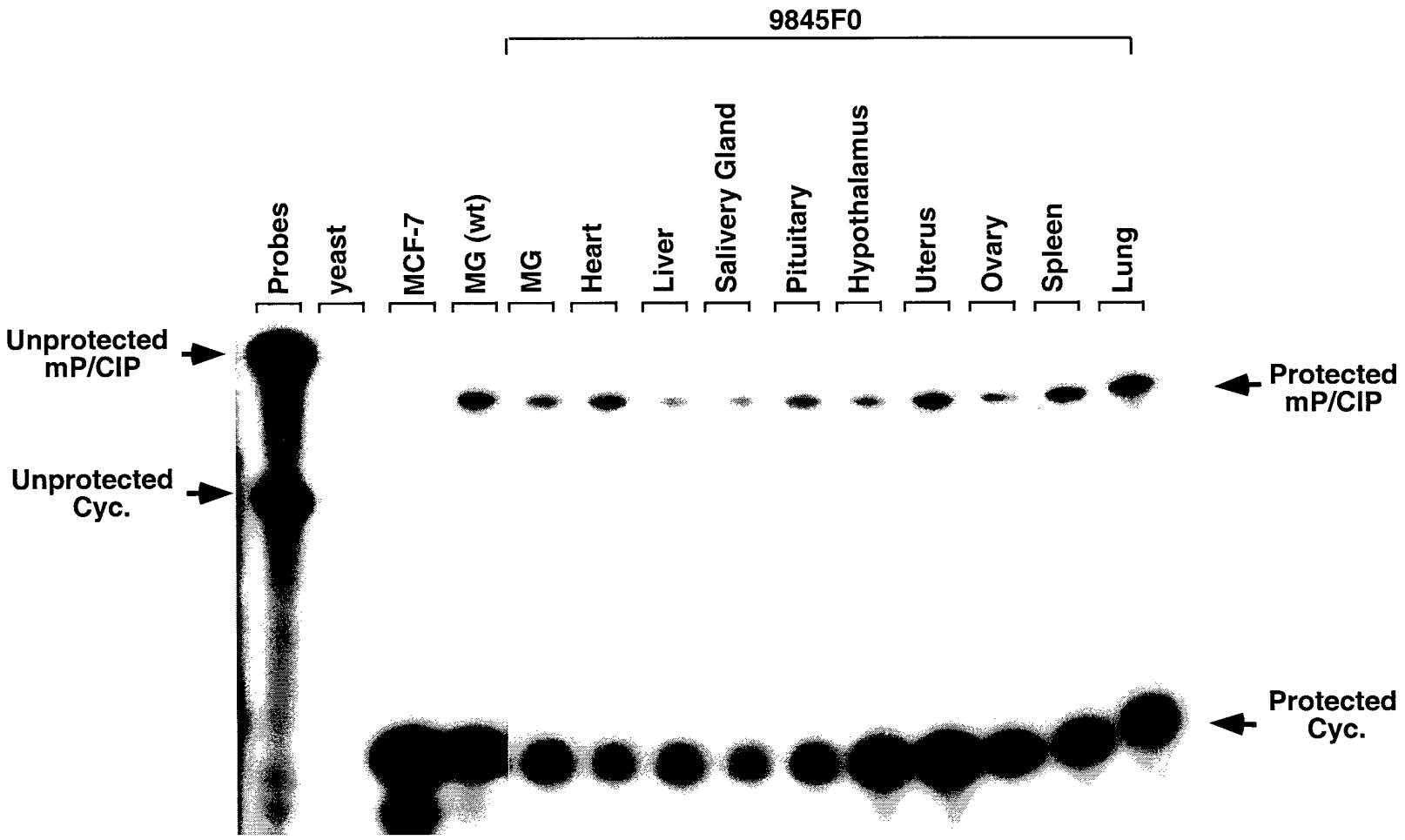
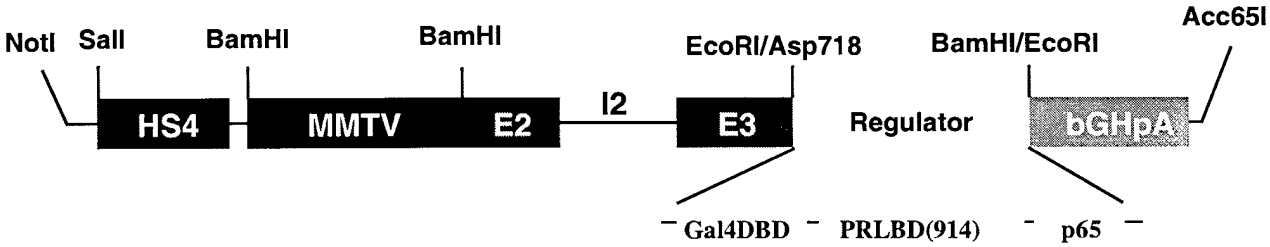


Figure 5

Regulable



Target Gene

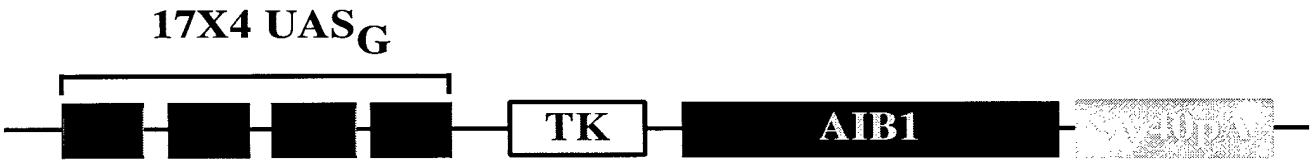


Figure 6

Tab. 1 Summary of MMTV-AIB1 Transgenic Screening

Transgenic Construct		No. of Pups Born	No. of Founder	Expression line
HM-AIB1	1. (*)	5	2	1 ($2649F_0$)
	2.	34	7	1 ($9845F_0$)
MS-AIB1	1.	11	4	0
	2.	44	3	n.d.

(*): Batch of Microinjection